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Heterogeneity of the mechanism of water splitting in photosystem II

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Abstract

We measured the temperature dependence of oxygen evolution in thylakoids from tobacco using mass spectrometry and high resolution polarography. We determined the initial S-state distribution and the efficiency of the transition between these states including the probability of the O_2 yield through a *fast mode*. We observed discontinuous changes of the parameters at the temperatures 11°C, 15°C and 21°C. Due to the mass spectroscopy data we think that the *irregularity* observed at 11°C is due to conformational changes within the water catalytic site. We show that the different contributions of the *slow* and *fast modes* of oxygen evolution and of the water molecule exchange are correlated and that their behavior can be explained in terms of the H_2O accessibility to the water splitting enzyme.

Keywords: Water splitting; Photosystem II; Mass spectrometry; Oxygen isotope; S-state temperature change

1. Introduction

Joliot et al. [1] observed that oxygen production in dark adapted chloroplasts, triggered by short light flashes, oscillated with a periodicity of four. The damped oscillations were described by Kok et al. [2], who proposed a linear four-step model for the water oxidizing cycle. It is commonly accepted that the oxygen evolving complex (OEC) accumulates successively four oxidizing redox equivalents in the manganese cluster [3]. Water molecules are split only when sufficient oxidizing power is accumulated. The water splitting enzyme is located on the lumenal side of the D1/D2 heterodimer surrounded by the extrinsic 33 kDa, 23 kDa and 17 kDa proteins [4]. In the absence of the external peptides oxygen evolution requires for proper functioning the addition of Ca^{2+} and Cl^-. However, the binding sites of the manganese cluster, calcium and chloride anions and their cooperation are not really known [5]. There are some models on the organization of the oxygen evolving complex in the literature [6]. However, they are not only based on the results of X-ray measurements [6], but also on EPR measurements which cannot be unambiguously interpreted [7]. Recently, it has been observed that four Mn ions are released and recombined pairwise. The different pairs of Mn exhibit different strengths of binding. Moreover, Mn dimers have been found to be exposed differently to the aqueous environment [8]. Despite extensive studies on the oxygen evolving complex, the problem of how, where and when hydrogen peroxide molecules or hydroxide species become included in the water splitting reactions is still open, hence the molecular mechanism of the water splitting process is not known. It is still under debate whether there is a

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specific mechanism, depending on the redox state of the manganese complex, which would trigger the water molecules' access to the catalytic site [9] or whether there is free access to the external solvent phase [10]. The fact that there are two different environments of Mn dimers [8] and our finding that a finite amount of water molecules is present in the cleavage site of the oxygen evolving complex [11] would rather support the latter hypothesis.

We apply, here, mass spectrometry and high resolution polarography to investigate the water splitting reaction. This allows us to follow directly the process of oxygen yield. Especially, temperature dependent measurements of oxygen evolution give better insights into the efficiency of processes governing the water splitting reaction. For example, the light-driven H_2O_2 decomposition under O_2 evolution in *Oscillatoria chalybea* depends on the temperature. When temperature decreases, the O_2 partial pressure, at which the decomposition of H_2O_2 gets inhibited, increases [12]. Temperature discontinuities of the initial S-state distribution and of the miss parameter, describing the redox states of manganese and the efficiency of their oxidation, respectively, have been observed for the cyanobacterium *O. chalybea* and *Chlorella kessleri* [13]. Some of the discontinuities have been seen in other measurements of redox components of photosystem (PS) II membrane fragments [14–20]. Discontinuities observed in temperature dependence of the fast transition, S_3 $(S_4) \rightarrow S_0 + O_2$ to the oxygen yield have been found at the same temperatures as changes of the other parameters within the 5S-state model [21]. In this model, the S_3-state can proceed in two different *modes* towards O_2 yield. The existence of these two ways is in agreement with the experimentally observed fast and slow rate of oxygen evolution in mass spectrometric studies [22]. The heterogeneous character of the OEC has been postulated for PS II membrane fragments of spinach already earlier [17] and for the thermophilic cyanobacterium *Synechococcus vulcanus* [14]. The heterogeneity of the S_3 transition may result not only from two different conformational states of S_3 but also from the heterogeneity of the secondary donor to PS II as well as from the non-homogeneous acceptor side of photosystem II [23–25]. It could be related to the organization of the water splitting enzyme, its accessibility to water molecules and/or a further charge stabilizing process allowing a more efficient electron extraction from H_2O.

This paper contributes to the clarification of the heterogeneity of the process of water splitting. We have measured the temperature dependence of oxygen evolution in thylakoids prepared from tobacco using mass spectrometry and high resolution polarography [26,27]. Redox states of the OEC and the effectiveness of transitions between them are determined according to the 5S-state model developed by Burda and Schmid [21]. The correspondence between the fast mode of oxygen evolution and two distinct ways of H_2O molecule interaction with the water splitting enzyme is discussed.

2. Materials and methods

Thylakoid samples were prepared from *Nicotiana tabacum* var. John William's Broadleaf (JWB) [28] with some minor modifications. Only freshly isolated thylakoids were used in our investigations. The samples were suspended in tricine (0.06 M)/KCl (0.03 M) buffer (pH 7.5). For measurements with the fast electrode system the sample contained thylakoids corresponding to 60 μg chlorophyll (Chl) and for mass spectroscopic studies 100 μg Chl. In the latter case 1 mM ferricyanide was added as exogenous acceptor.

Amperometric measurements of oxygen evolution under short saturating flashes were carried out with the *three-electrode system* described by Schmid and Thibault [26]. The polarization voltage was −680 mV. Flashes were provided by the Stroboscope 1539A from General Radio (xenon flash lamp) with a flash duration of 5 μs at half intensity. Usually, 15 flashes spaced 300 ms apart were given. The electrode system was connected with a thermostat. The temperature was stabilized within ± 0.1°C.

The sample was kept in ice in darkness before putting it on the electrode. The handling was performed in dim green light. The thylakoids were incubated in darkness at the respective temperature for 15 min (the sample volume was 600 μl).

Mass spectrometric measurements were performed by means of a modified magnetic sector field spectrometer 'type Delta' from Finnigan MAT (Bremen, Germany). The details of the adapted setup for highly sensitive photosynthetic experiments have

been described earlier [10,12]. 32 ($^{16}O_2$), 34 ($^{16}O^{18}O$) and 36 ($^{18}O_2$) signals were simultaneously detected in Faraday cups and recorded on a SE 130-03 BBC Metrawatt three-channel recorder. The holder of the sample was cooled via connection with a thermostat, stabilizing the temperature within ±0.1°C. Every 10 min, after $H_2^{18}O$ injection, 10 short saturating flashes (as in the case of polarographic studies) were given. $H_2^{18}O$ was obtained from the CEA-Oris, Bureau des Isotopes Stables (Gif-sur-Yvette, France).

3. Results

The polarographic measurements of oxygen evolution were carried out in the temperature range of 6–25°C. Oscillations of the O_2 yield pattern improved with decreasing temperatures. The data were analyzed using the 5S-state model (S_0, S_1, S_2, S_3, (S_4)) assuming two ways of oxygen evolution [21] (Fig. 1). Parameter d used in the model corresponds to the fraction of the fast transition S_3 (S_4)$\rightarrow S_0+O_2$ whereas $1-d$ is the fraction of the O_2 yield due to the *slower* way or *mode* passing through a longer living S_4-state. The temperature dependence of the initial S_i-state ($i=0$, 1, 2, 3) distribution is shown in Fig. 2A,B. As is expected [29,30] the most occupied state is S_1. The S_2-state is only slightly populated and S_3 is almost empty. There are three characteristic temperatures at which the initial distribution of the S_0- and S_1-states are discontinuous, namely: 11°C, 15°C and 21°C (Fig. 2A). The S_1-state population shows a tendency to increase with decreasing temperatures starting from 11°C, whereas the S_0-state decreases at the same time. The S_2- and S_3-states increase for temperatures below 15°C (Fig. 2B). The total miss parameter α_t is the sum of all misses $\Sigma_{i=0,1,2,3}\ \alpha_i$ for separate transitions $S_i \rightarrow S_{i+1}$. At temperatures above 15°C, the main contribution to α_t comes from the probabilities of failure for the transitions $S_0 \rightarrow S_1$ and $S_2 \rightarrow S_3$. Below 15°C the miss parameter α_t decreases significantly (Fig. 3A). The increase of the d parameter is accompanied by a decrease of α_t with decreasing temperatures (Fig. 3B). These two parameters are characterized by the same temperature discontinuity as the S-states. The most pronounced

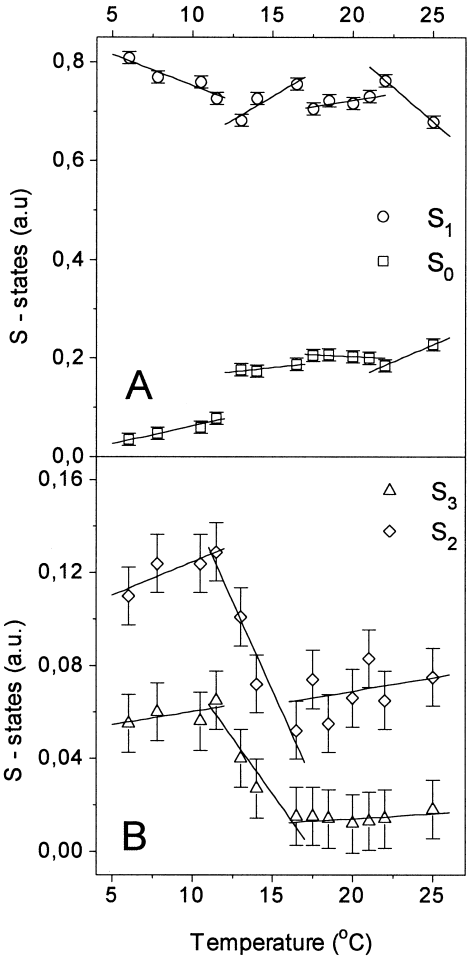

Fig. 2. Temperature dependence of the initial S-state distribution. (A) The S_0- and S_1-states and (B) the S_2 and S_3 states in *N. tabacum* var. JWB thylakoids enriched in PS II. The S_i-states are calculated from the heterogeneous 5S-state model. Maximal error bars are given in the figures.

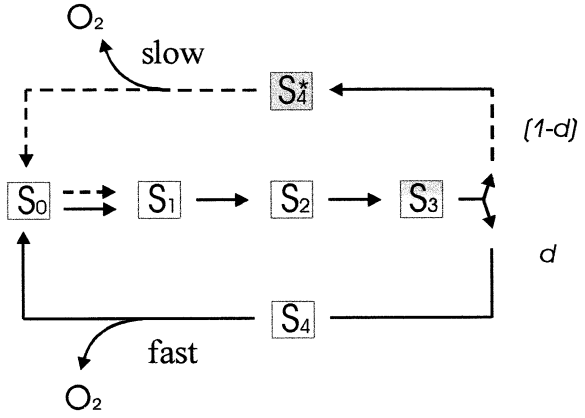

Fig. 1. Scheme of the two ways of oxygen evolution. d, fraction of the fast channel of O_2 yield; $(1-d)$, fraction of the slow channel of O_2 yield.

changes of d and α_t occur within the range of 11–15°C.

Fig. 4 shows the dependence of photosynthetic oxygen evolution measured as $^{18}O_2$, $^{16}O_2$ and the mixed isotope molecule $^{18}O^{16}O$ on the temperature. Each data point represents an average over 14 measurements taken at a given temperature. The measurements have been done when a steady state of $H_2^{16}O/H_2^{18}O$ redistribution in solution after the injection of labeled water is achieved. At lower temperatures the steady state is reached within 70 min. The data shown in Fig. 4 were collected starting from 100 min after the $H_2^{18}O$ injection. The sample was flashed every 10 min. The assay contained 1 ml 95.7% $H_2^{18}O$ which corresponds to a p-parameter of enrichment equal to 0.319. The theoretically expected isotope

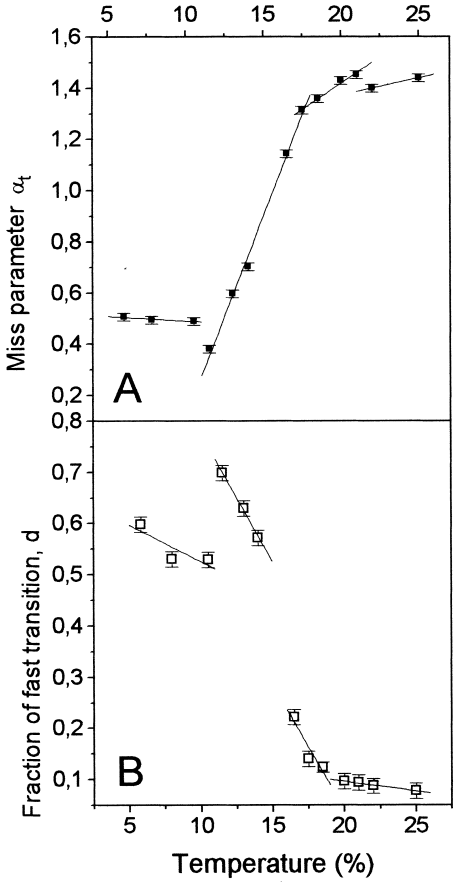

Fig. 3. The total miss parameter α_t (A) and the probability d of a fast transition $S_3 \rightarrow (S_4) \rightarrow S_0 + O_2$ as the function of temperature in *N. tabacum* var. JWB thylakoids enriched in PS II. The parameter is designated in the ordinate of the figure as the fraction of fast transitions. Error bars are given in the figure.

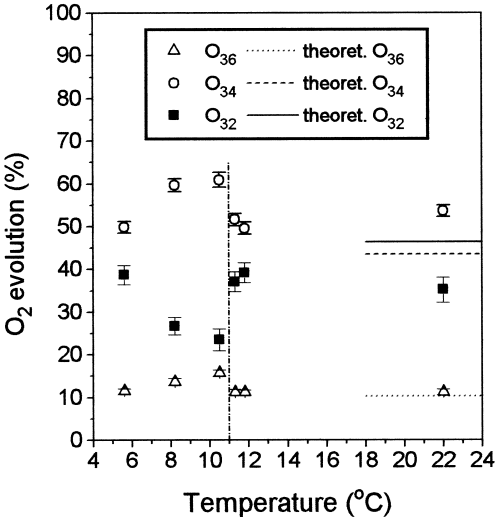

Fig. 4. Temperature dependence of photosynthetic oxygen evolution of $^{18}O_2$, $^{16}O_2$ and the mixed molecule $^{16}O^{18}O$ in *N. tabacum* var. JWB thylakoids enriched in PS II. The reaction buffer was enriched in 31.9% $H_2^{18}O$. Error bars are given in the figure as well as the level of the expected values of the oxygen species $^{16}O_2$, $^{16}O^{18}O$ and $^{18}O_2$.

distribution between $^{18}O::^{18}O$, $^{18}O::^{16}O$ and $^{16}O::^{16}O$ is $36:34:32 = p^2:2p(1-p):(1-p)^2$ [10]. In our case, for p given above, the relative amounts of the O_2 yield should be 10.2%, 43,4% and 46,4% for the signals at $m/e = 36$, 34, and 32, respectively. In the experiment we have observed that signals 36 and 34 are higher than the theoretical ones, while the 32 signal is lower. This effect is discussed in [11]. The striking feature of the curves showing the dependence of the oxygen signals on temperature is the presence of a discontinuity/jump at 11°C. We think that the discontinuity has its origin in the reorganization of the water splitting enzyme, because we observed the transition at the same temperature for other enrichments.

4. Discussion

The discontinuities of the initial S-state distribution, of the miss parameter α and of the d parameter at 11°C, 15°C and 21°C for tobacco thylakoids are exactly at the same temperatures as the ones for *C. kessleri* and *O. chalybea* reported earlier [13]. The sharpness of O_2 oscillations improves when the temperature decreases. This follows from the increase

of the fast transition fraction of oxygen yield and the simultaneous decrease of non-successful transitions between the $S_i \rightarrow S_{i+1}$ states. Similar effects have been observed for *Chlorella* and *Oscillatoria*. It is clear that at these lower temperatures new equilibria of redox states on the donor and acceptor side of photosystem II are established. The particular temperature range where it happens may differ from species to species with the final effect being the same, namely that at lower temperatures the fast mode of oxygen evolution becomes as important as the slower one. Thus at 20°C for tobacco thylakoids the *d* parameter is less than 0.1 whereas at 10°C it is about 0.55 (Fig. 3B). A similar dependence of the *d* parameter can be expected for thylakoids prepared from other higher plants. For the cyanobacterium and algae the temperature scale differs slightly, but the slow phase of the oxygen yield is dominant at temperatures $\geq$25°C ($d < 0.1$). This is probably due to the fact that their optimal growth conditions require higher temperatures (26–30°C) in comparison to tobacco. Cyanobacteria also differ in their operating of the OEC in comparison to higher plants. We want to emphasize that the 5S-state model naturally explains the experimental data for the 34-O_2 yield time dependence for different temperatures known from the literature [31]. The process takes place as two independent relaxations separated in their time scales. Mathematically, the amplitude of such a double relaxation is given by the sum of two exponential relaxations with a total amplitude normalized to unity:

$$\text{Signal}_{34} = A(1-\exp(-k_1 t)) + (1-A)(1-\exp(-k_2 t) \quad (1)$$

For each temperature, there are the three independent parameters A, k_1 and k_2. In practice, we see that the freedom in the parameter A plays the crucial role for the quality of the fit and allows the fits to be flexibly adjusted to the data points from the experiments mentioned above [31]. The experimental data of these authors seemingly show that the amplitudes for the slow and fast water exchange are practically the same and that this is valid under different conditions, e.g. temperature. However, their amplitude calculations depend on the $H_2^{18}O$ enrichment. The analysis presented here is independent on the fraction of labeled water. Our parameters are collected in Fig. 5. It appears that the parameter A changes with the temperatures in a similar way as the parameter d obtained from the 5S-state model. The comparison of the temperature change of the amplitude of the fraction of fast exchangeable water molecules with the fast mode of the O_2 yield obtained from the 5S-state model clearly shows that they are correlated (Fig. 5). They increase with decreasing temperatures.

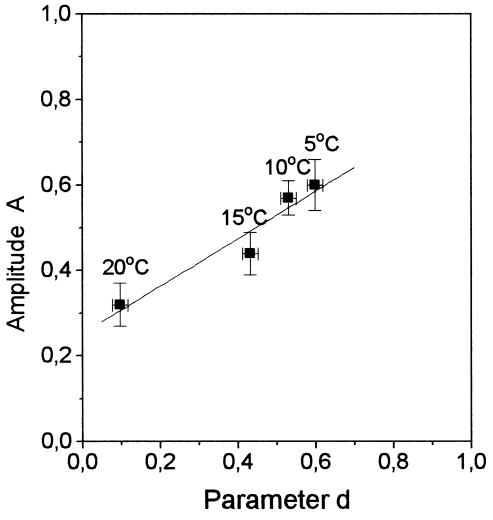

Fig. 5. Correlation between parameter d of the fast transition $S_3 \rightarrow (S_4) \rightarrow S_0 + O_2$ and parameter A of the amplitude of the fast phase (from Eq. 1).

It is difficult to assign the temperature transitions of the parameters S_i, α_t and d to particular changes of the oxygen evolving complex, an exception being the transition at 11°C which can be clearly attributed to a new arrangement of the catalytic site of the water splitting enzyme. The decrease of the initial S_0 population at 11°C and the increase of the higher states S_2 and S_3 result from different conformations of the water splitting enzyme. Mass spectroscopic measurements, which show a significant temperature discontinuity of $^{18}O_2$, $^{16}O^{18}O$ and $^{16}O_2$ evolution at 11°C, corroborate this view (see Fig. 4). This implicates that the different contributions of the slow and fast modes of water exchange and of O_2 evolution originate from the temperature dependent water accessibility to the water splitting enzyme, probably as a result of its structural rearrangement.

The redox active tyrosyl residues TyrD and TyrZ have been shown to be located toward the lumenal side of PS II. The environment of TyrD is hydro-

phobic but the surrounding of TyrZ has been found to be much more hydrophilic because it is in the vicinity of polar and charged residues [32–34]. Therefore there is no reason why H_2O molecules might not be present close to the water splitting enzyme and we conclude that the pocket of the oxygen evolving complex is not 'dry'. The conformational changes of the OEC pocket, which depend on temperature, may influence the amount of water molecules entering the pocket. Additionally, the properties of the electron transfer chain change with temperature because of rearrangements of integral proteins and/or because the polar lipids take part in the stabilization of new dark and light redox equilibria within the studied temperature range [35–37]. The low occupation of the S_0-state at temperatures below 11°C is most probably caused by the acceleration of TyrD reduction by the S_0-state [30,38,39]. At the same time the S_2- and S_3-states become more stable, which can be explained by an inhibition of the back reactions from Q_A^- since a significant *decrease* of misses is observed at these temperatures. However, the stabilization of reduced Q_A^- is not consistent with the enhanced efficiency of oxygen evolution. Therefore we rather think that the reduced $TyrD^{red}$, which is known to be oxidized by the S_2- or S_3-state within a few seconds [39,40], is more stable at lower temperatures. Of course, the TyrZ surrounding and that of the manganese complex itself affect the initial distribution of S-states. The heterogeneity of the water splitting mechanism can be attributed to a distinct accumulation of oxidizing power (oxidation of Mn and/or His during the $S_2 \rightarrow S_3$ transition) [41,42]. The different S_3-states can bind substrate water in different ways and as a result two modes of oxygen evolution are observed. For the functional heterogeneity of the manganese cluster, an asymmetric protein matrix around the Mn complex can be responsible [43]. The process of the storage of four oxidized equivalents may also be influenced by other components of the photosystem II, such as cytochrome *b*-559 [44], CP47 [45,46] or CP43 [47].

5. Conclusions

We have shown for tobacco thylakoids that the initial S-state distribution and the efficiency of transitions between these states and the fraction of fast O_2 yield exhibit characteristic temperature transitions at 11°C, 15°C and 21°C. The temperature discontinuity at 11°C apparently comes from conformational changes within the water splitting enzyme. The decrease of S_0 and the increase of S_2 and S_3 populations with decreasing temperatures can be related to the acceleration of the $TyrD^{ox}$ reduction and the $TyrD^{red}$ stabilization. We have shown that the fast and slow exchanging substrate water molecules correspond to the fast and slow modes of oxygen evolution, respectively. The heterogeneity of the water splitting enzyme, i.e. how four oxidizing equivalents are stored, affects the substrate water binding and through this the mode of oxygen yield. In particular, the heterogeneity of the S_3-state seems to play the role of a switch between the two channels of O_2 evolution. Two distinguished environments of Mn dimers may be responsible for the heterogeneity.

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